Bead-based multiplex ELISA

for quantification of Albumin concentration in urine samples

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Abstract

In this paper, we discuss the use of a variant of the enzyme-linked immunosorbent assay (ELISA) protocol for diagnostic purposes. We performed the bead-based multiplex ELISA method, in order to quantify the concentration of Albumin in urine samples. Our goal is to test subjects for albuminuria, which could indicate Chronic Kidney Disease.

Intro

The enzyme-linked immunosorbent assay (ELISA) is a well-established method for detecting and quantifying "analytes", i.e. substances such as proteins, antibodies, peptides and hormones, in liquid samples.

This technique makes use of an antigen and its binding antibody (one or more), which is linked to an enzyme. This enzyme reacts with an added substrate, to produce a detectable signal, e.g. color change.

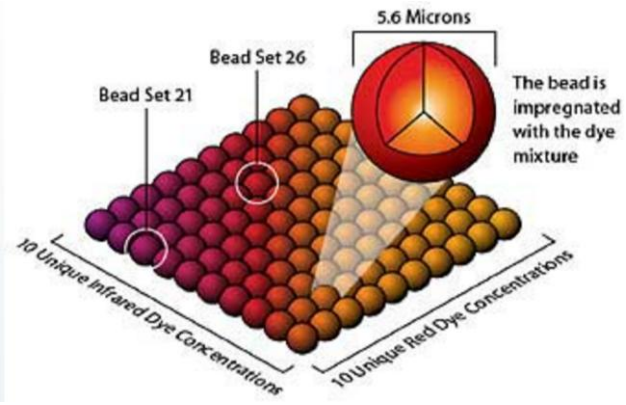
There are different variants of the technique, namely:

- Direct ELISA
- Indirect ELISA
- Competitive ELISA
- Sandwich ELISA

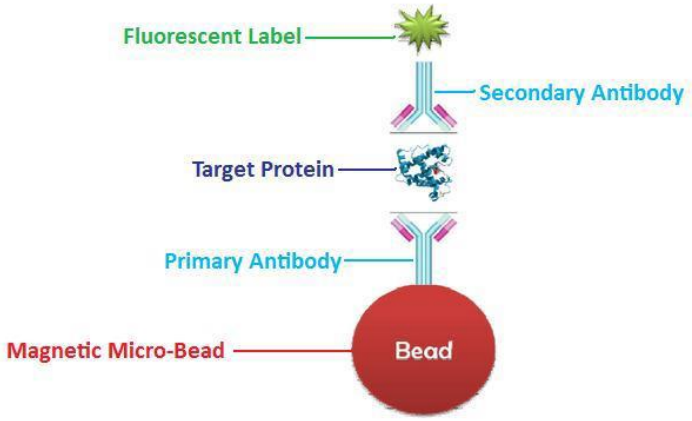
Experiment Procedure

In the discussed experiment, we conducted the **Bead-Based Sandwich ELISA**, which allows **multiplexing**, meaning the simultaneous measurement of multiple analytes in a single run/cycle of the assay. The steps necessary are presented here:

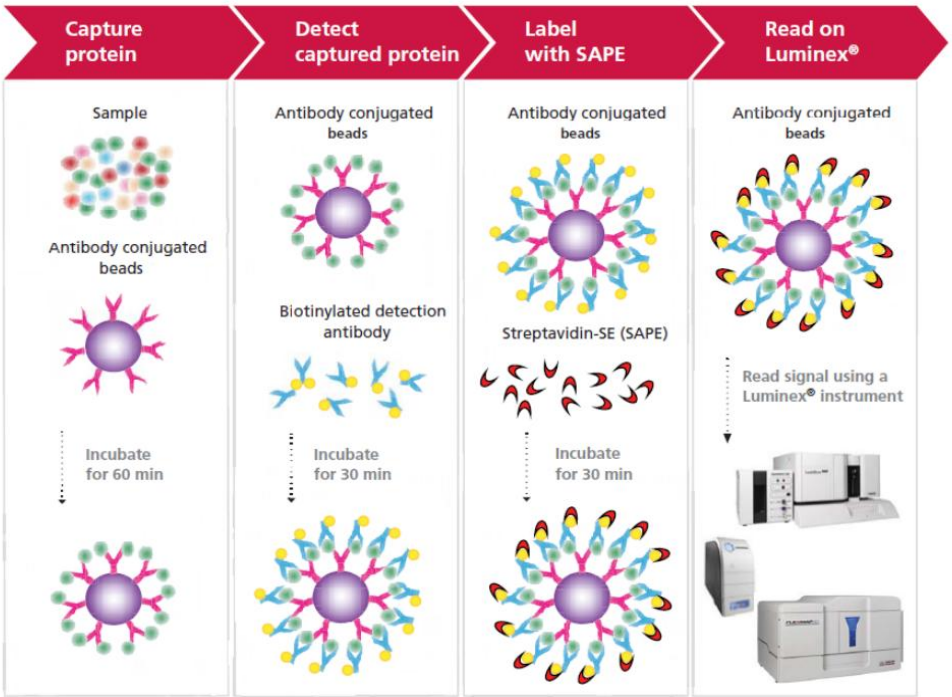
1. This procedure makes use of magnetic beads that attach to the bottom of the well. They are color-coded, with red and infrared dye in different concentrations, which enable multiplexing. These beads are linked to antibodies, called "capture antibodies" that are specific to the antigen of interest.



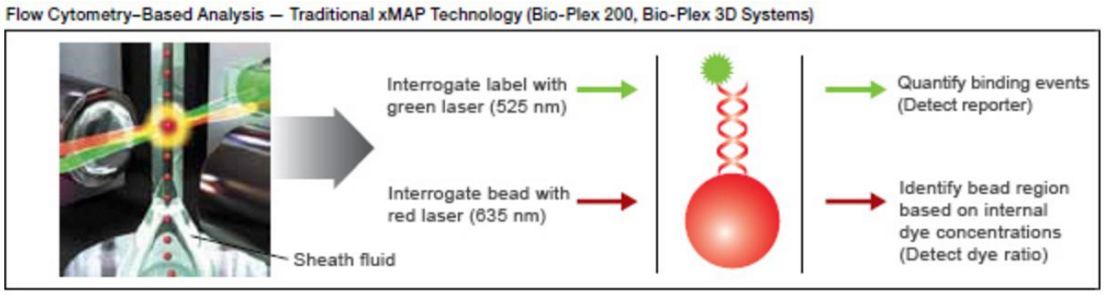
2. By adding the sample we want to test, these antigens (if present) will bind with the antibodies inside the well. In this stage, the antigen of our choice is “captured” on the bottom of the well.
3. Subsequently, we add a second antibody, called “detection antibody”, which is *biotinalyted*, in order to facilitate detection, as the name implies. This secondary antibody is also specific to the analyte of interest; therefore it binds with the antigen, forming a “sandwich” of two antibodies, with the antigen in the middle.



4. In the next step, Phycoerythrin (PE)-conjugated Streptavidin (SAPE) is added. This molecule binds with biotin linked to the second antibody, labeling the “sandwich” mentioned above. PE is fluorescent, enabling detection upon excitation.



5. Finally, the results are obtained (usually) through a double-laser system. A thin needle is inserted in the well and draws part of the solution. Its diameter permits the passing of only one bead at a time. The two lasers excite both the bead and PE (if present), which in turn emit light at a different (known) wavelength. Sensors detect the emitted light's intensity and wavelength. The wavelength emitted from the bead tells us the kind/type of the bead, and the intensity of the light coming from PE informs us about the concentration of the antigen (if present). Usually, this is done using a LUMINEX Instrument that saves and processes acquired data in a computer.



We must note that, between each step of the process, any liquid inside the well is disposed and the wells are "washed" properly. The magnetic nature of the beads holds them in place, in the bottom of the well, and therefore any antibodies and antigens bound to the beads are also held in place, between washes.

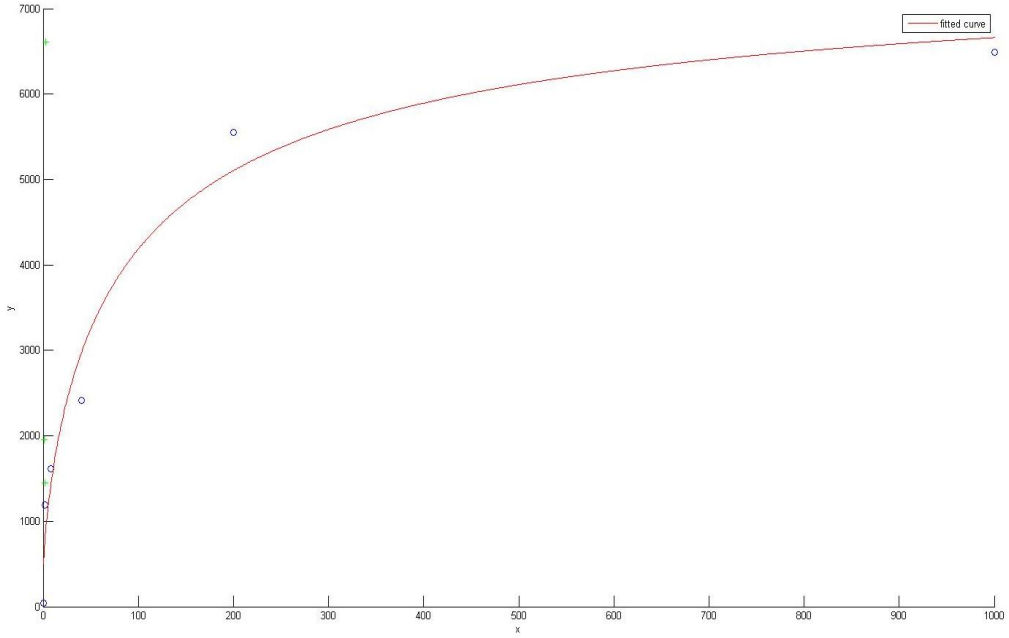
Another element that is crucial to the process is the incubation time needed between each step, so that antigen-antibody bonds are created and beads are attached to the bottom. The time needed between each step is described in the protocol of the assay.

Results

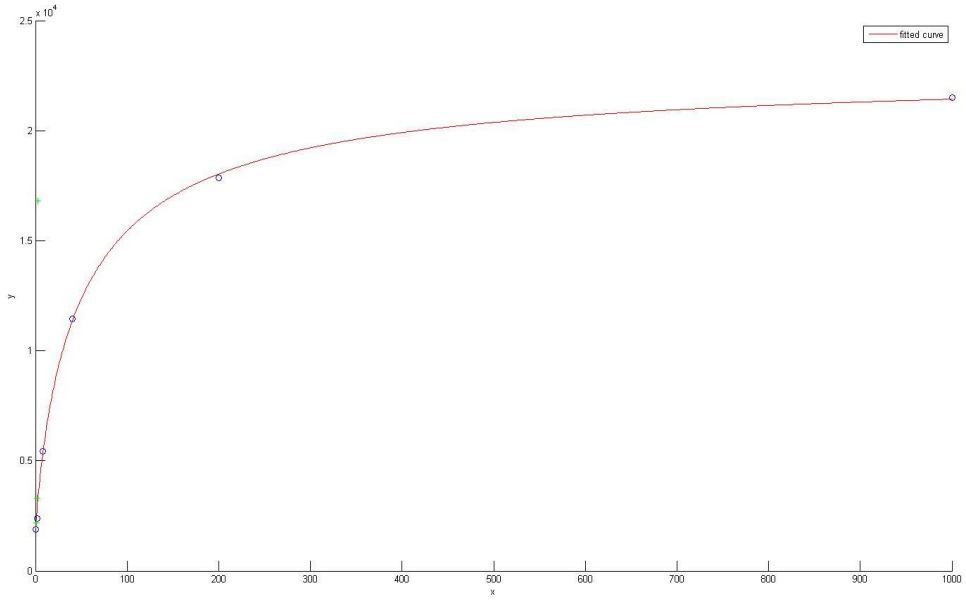
From the Luminex machine we receive a measurement of the Net MFI(Median Fluorescent Intensity) of each sample, which then needs to be matched with a corresponding concentration of albumin in that sample. To achieve that, we create two calibration curves (one for each type of bead) by using wells H1 through H6, where we have deposited pure albumin in concentrations of 1000, 200, 40, 8, 1.6, and 0 μg/ml respectively. After receiving the Net MFI of these cells from the Luminex, we use the 4PL method to construct the required curves, as follows:

- the X-axis is defined by the vector of the above concentrations in wells H1-H6 X=[1000 200 40 8 1.6 0]
- the Y-axis is defined by the corresponding Net MFI values for each of the bead types in the same wells.
- the above values are used with the Matlab file L4P.m (found in Mathworks site) to calculate the coefficients of the 4PL curves
- the two curves are then plotted using Matlab

Analyte 26



Analyte 37



After plotting the curves, we place the samples' Net MFI values on the Y-axis of the graphs for each of the analytes, and find the corresponding albumin concentration Value on the X-axis. The results are as follows:

Analyte 26	Net MFI (Median)	Albumin concentration (ng/ml)
Patient 1	1953	16000
Patient 2	1452	8000
Patient 3	6607	942000

Analyte 37	Net MFI (Median)	Albumin concentration (ng/ml)
Patient 1	2175	1000
Patient 2	3299	3000
Patient 3	16793	140000

Comments

From the diagrams after the curve fitting and the analysis, for the two different kinds of beads and the three different samples, it is evident that the first two samples from presumably healthy subjects have quite low MFI values as expected.

In contrast, the sample from the patient with Chronic Kidney Failure shows significantly higher levels of MFI, higher by 4500 values in the first analyte used (analyte 26) and by 13000 in the analyte 37.

We can see that the first analyte produces a slightly worse curve than the one produced by the second analyte and there is a difference of approximately 10000 MFI values regarding the patient between the two beads used (6500 for analyte 26 and 16000 for analyte 37). Even though the results may vary when using different kinds of beads, the main conclusion is the same, as the patient shows a lot higher values than the other two samples of hypothetically healthy subjects.

The results confirm the condition of the patient, who was selected for the testing and experimentation with the ELISA method. The bead-based multiplex ELISA method produced quite accurate and fast results, minimizing time and cost. It can be used as a basis for more detailed screening tests.

Albuminuria and CKD

Albuminuria is a pathological condition wherein the protein Albumin is found in high levels in the urine. Sometimes is referred to as microalbuminuria, when Albunmin lies between 30 and 300 mg/24h, mg/l of urine or μg/mg of creatinine.

Chronic and persistent albuminuria is a result of kidney failure, meaning gradual loss of kidney function. The damaged kidney starts to spill some albumin into the urine, leading to Chronic Kidney failure. When chronic kidney disease reaches an advanced state, dangerous levels of fluid and wastes build up in your body.

Other causes of albuminuria are high blood pressure, congestive heart failure, the metabolic syndrome or kidney damage from nephrotic syndrome. Generally, having high levels of albumin in the urine is a risk factor for cardiovascular disease. Not all patients conditioned with albuminuria can be identified with kidney disease. However, in patients with diabetes or established chronic kidney disease, albuminuria is associated with more rapid progression of their chronic kidney disease and a greater chance that kidney failure will develop.

About 8 % of adults have microalbuminuria, and a quite small percentage (1%) has macroalbuminuria. Albuminuria was detected in one of every three persons with diabetes, one of every seven persons with high blood pressure but no diabetes, and one of every six persons older than 60 years.

In early stages of chronic kidney disease the symptoms may be few and scarce and it may not become apparent until the kidney function is significantly impaired.